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RAPID DETECTION OF MYCOBACTERIA IN PATIENTS
WITH HIV INFECTION

ANNUAL REPORT



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The Polymerase Chain Reaction (PCR) along with hybridization to chemilumenescent DNA probes was used to detect Mycobacteria potentially present in patient specimens from the Mycobacteriology laboratory at Walter Reed Army Medical Center (WRAMC). DNA from the specimens were prepared by two different methods, and used in the PCR amplifications. Of the specimens tested, 88% were known to contain Mycobacteria. Of these, we were able to detect 20 (54%), though we were able to detect 82% (9 of 11) using a new genus specific primer set. With our M. tuberculosis primers, we were able to detect 21 of 28 samples that were positive by culture results (75%). We are continuing to evaluate these primer sets, as well as trying to design a primer set for M. avium.

RA 1; Mycobacteria; Mycobacterium avium; Mycobacterium tuberculosis; Polymerase Chain Reaction; DNA probes, Rapid identification

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David C. Fritz 10 May 1993
Principal Investigator's Signature Date

## INTRODUCTION

Mycobacterial infections are being recognized as being a major problem in patients with compromised immune systems, such as those with AIDS (Collins, 1992). The two most common Mycobacterial species found in AIDS patients are *M. tuberculosis* and *M. avium*, though opportunistic infections caused by other Mycobacterial species have been reported. While infection by *M. avium* seems to be an opportunistic infection, occurring in the late stages of clinical AIDS (Collins, 1992), infection by *M. tuberculosis* takes often occurs much earlier in the course of the disease. Indeed, there appears to be a synergistic effect between the two types of infection, since the onset of tuberculosis can accelerate the onset of clinical AIDS, reducing the survival time to less than eight months (Horsburgh and Silik, 1989). For these reasons, it is very important that Mycobacterial infections in HIV infected patients be diagnosed as rapidly as possible.

Detection of Mycobacteria in clinical samples is hampered by the slow growth rate of the bacteria (from 4 to 8 weeks to grow a culture of *M. tuberculosis*), and by the difficulty in differentiating different species of acid-fast bacilli in samples (Sommers and Good, 1985). Shortening the interval required to diagnose Mycobacterial infection, and to differentiate among Mycobacterial species is clearly of major importance. One method that has been used is the Polymerase Chain Reaction (PCR), using primers specific for Mycobacterial DNA sequences ( Böddinghaus, et al, 1990; Kolk, et al, 1992; Shawar et al, 1993; Scini et al, 1992; Thierry, et al, 1992).

Previously, Dr. Patel isolated sequences that were specific for Mycobacteria (Fries et al, 1990), for *M. avium* (Fries et al, 1990), and for *M. tuberculosis* (Patel, et al, 1989; Patel et al, 1990) and designed primers for each. For the last two years, she was using these primer sets to screen samples provided by the Walter Reed Army Medical Center for Mycobacteria, and to distinguish between *M. avium* and *M. tuberculosis*. After she left last June, her technician and Dr. Hadfield tried to repeat her results, with little success. In summary, we found that her primers did not appear to be specific enough, often amplifying a number of different targets in both purified Mycobacterial DNA, and in DNA isolated from clinical samples. I arrived in January, 1993, and have attempted to overcome these problems by, first, using computer analysis to design new primers from Dr. Patel's species specific sequences, and, second, by finding new sequences that are genus or species specific, and using computer analysis to design primers for the specific amplification of these sequences for the detection of Mycobacteria in clinical samples. The results of this work are described below.

## MATERIALS AND METHODS

### Materials:

All materials for DNA amplification were purchased from Perkin Elmer (Norwalk, CT). Agarose was purchased from Gibco/BRL (Gaithersburg, MD). Oligonucleotides used for PCR primers and hybridization probes were synthesized on a Applied Biosystems 380B DNA synthesizer. DNA labeling kits (Genius™ 6) were purchased from Boehringer Mannheim (Indianapolis, IN). All other chemicals were reagent grade or better.

## Methods:

**Preparation of Clinical Samples:** DNA was isolated from Clinical samples by the method of Boom et al (1990). Briefly, in this method, the sample is added to a lysing solution containing 9 M guanidinium isothiocynate and vortexed in the presence of glass beads. The high concentration of the guanidinium salt lyses the cells and causes the DNA to bind to the glass. The beads are then washed with a second guanidinium containing buffer, with ethanol and finally with acetone. The beads are then dried, and the DNA eluted with water or TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA).

DNA from clinical samples have also been prepared by the method of Thierry et al (1992). In this procedure, 0.5 to 1 ml. of sample was pelleted and resuspended in 200 µl of lysis buffer (0.1 M NaOH, 2 M NaCl, and 0.5% SDS) and incubated at 95°C for 15 minutes. The sample was then extracted twice with phenol-chloroform, and ethanol precipitated. The DNA was resuspended in 100 µl of water, and 10 µl aliquots were used for the amplification reactions.

**Amplification and Detection of Mycobacterial DNA in Clinical Samples:** Mycobacterial DNA was amplified in a standard 100 µl reaction mix containing: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, approximately 2 mM $MgCl_2$ (depending on the optimal $MgCl_2$ determined for the primer set used), 1 mM in each dNTP, 1 µM in each primer, 25U/ml AmpliTaq® DNA polymerase, and either 0.1 ng control DNA, or 5-10 µl of DNA from a clinical sample. The timing and temperatures of the thermal cycling reactions were dependent on the primer set used. In all cases, controls were run that were -DNA and that contained purified *M. avium* and *M. tuberculosis* DNA.

One tenth volume of each reaction was electrophoresed on a 1.8% agarose gel, that was then stained with 0.5 µg/ml ethidium bromide. After staining, the samples were transferred to nylon filters by the method of Southern (1975). Oligonucleotide probes were 3' end labeled with digoxigenin-11-dUTP (DIG-dUTP) by tailing with terminal deoxynucleotide transferase, following the instructions in the Genius™ 6 kit. Briefly, approximately 100 pmole of the oligonucleotide is tailed in a 20 µl reaction mix containing 200 mM sodium cacodylate, 25 mM Tris-HCl (pH 6.6), 0.25 mg/ml BSA, 5 mM $CoCl_2$, 50 µM Dig-dUTP, 500 mM ATP, and 2.5 U/µl terminal transferase. After an incubation of 15 minutes at 37°C, the reaction was put on ice and stopped by the addition of EDTA. The reaction products were then ethanol precipitated in the presence of glycogen. Hybridization was carried out as described in the Boehringer Mannheim Genius™ System Users guide for Filter Hybridization, though at different temperatures and formamide concentrations, depending on the oligonucleotides used as probes.

## RESULTS

The ability of primer sets to detect Mycobacteria was evaluated in several different ways. First, primers were tested for their ability to amplify DNA of the correct genus and species. Primers that specifically amplified DNA from the correct species were tested for their sensitivity to detect small quantities of Mycobacterial DNA, so that we could determine the minimum number of bacteria that can be detected in a clinical sample (either by the ethidium bromide stained gel assay or by hybridization). For example, it is known that a single Mycobacteria contains about 5 fg of DNA, meaning that the ability to detect 10 fg of DNA would be equivalent of 2 bacteria in a sample. Then, the primers were tested for their

ability to detect Mycobacterial DNA in previously tested, known positive clinical samples. Finally, the primer sets will be evaluated using unknown clinical samples.

As described earlier, the primer sets used by Dr. Patel proved to be inefficient and unreliable in detecting Mycobacterial DNA in clinical samples. Computer evaluation of the Mycobacterial genus specific sequence and of the *M. avium* specific sequence did find other primer sets that appeared to be usable, while no suitable primer sets were found for *M. tuberculosis*. DNA titration analysis of the primers showed that the genus specific primer set was able to detect 1 pg of Mycobacterial DNA by gel analysis (equivalent to about 200 bacteria), and about 100 fg (20 bacteria) of DNA by hybridization analysis, while the *M. avium* specific primers were able to detect 10 pg of DNA by the gel assay, and 1 pg by hybridization. Though this was not deemed to be sensitive enough to detect Mycobacteria in clinical samples, we used these primer sets to screen clinical samples while we looked for more suitable primer sets.

For the detection of *M. tuberculosis*, a primer set from Shawar et al (1993) was tested. This primer set is derived from the sequence from the *M tuberculosis* insertion sequence IS6110, giving a fragment of 317 bp. When the new *M. tuberculosis* primer set was analyzed by DNA titration, it was found to be easily sensitive to less than 1 fg (0.2 bacteria) of *M. tuberculosis* DNA, both by gel analysis, and by hybridization. This was the primer set that we used for testing clinical samples for *M. tuberculosis.*

To find a DNA sequence suitable for the detection of all Mycobacteria, we decided to try a set of primers based on the sequence of the 16S ribosomal RNA, since sequence analysis of all Mycobacterial 16S rRNAs show that they are greater than 95% identical in sequence, while comparison of their sequences to other bacterial 16S rRNAs show more significant differences (Rogall et al, 1990). Since the differences are clustered in certain regions, it was decided to choose one of these non-homologous regions as one primer, and to use computer evaluation to find another suitable primer. The two primers chosen were 18mers starting at position 424 for the positive strand primer, and at position 887 for the negative strand primer, giving a product of 441 bp. Evaluation of this primer set showed that it was specific for Mycobacteria, and that it was able to detect approximately 1 fg of purified Mycobacterial DNA, both by gel analysis and by hybridization. This is the sequence that we will be using for detection of Mycobacteria in clinical samples.

A survey of the DNA databases showed only a single *M. avium* sequence, that of the insertion sequence IS901 (Kunze et al, 1991). We have used computer analysis to find several primer sets from the IS901 sequence. Unfortunately, while all the primer sets found to date have amplified only *M. avium* DNA, they have only been able to detect *M. avium* DNA down to the picogram level, which is not considered sensitive enough for these assays. Therefore, we are continuing to search for more primer sets from the IS901 sequence, as well as looking for methods for finding other *M. avium* specific sequences that could be used for amplification.

To evaluate the ability of the first three primer sets to detect Mycobacterial DNA in clinical (sputum) samples, we tested them against 42 different samples containing known Mycobacteria. These results are shown in Table 1, and summarized below. Of the 42 samples, 37 were known to contain Mycobacteria, either by culturing the bacteria from the samples, or by testing the cultures using the GenProbe® kit that is commercially available. Of these 37 Mycobacterial positive samples, 29 contained *M. tuberculosis*, 3 contained either *M. avium* or *M. intercullare*, while the remaining 5 contained other Mycobacterial species. By gel assay, the genus specific primers were able to detect Mycobacterial DNA in 9 samples (24%), with no false positives. Hybridization analysis increased the number of samples found to contain Mycobacteria to 20 (54%), again with no false positives. The

*M. avium* primers were not able to detect *M. avium* in any of the samples tested by gel analysis, but were able to detect *M. avium* in 2 of the 3 culture positive samples (67%). It should be noted that, because of the lack of sensitivity of the *M. avium* primers, they were not used to assay a number of these samples, and that other *M. avium* primer sets are currently being examined for their utility in detecting *M. avium*. The *M. tuberculosis* primers proved to be much more sensitive in detecting Mycobacterial DNA in clinical samples, correctly identifying 19 samples as *M. tuberculosis* positive by the gel assay (66%), and 22 samples as positive by hybridization (76%). The sensitivity of the *M. tuberculosis* primer set did prove to be a problem at times, in that great care needed to be taken not to contaminate negative samples, either before the PCR reaction, or prior to running the gels. We have also been able to obtain some preliminary data on the 16S rRNA derived genus specific primer set. When tested on clinical samples (Table 2), it was able to detect Mycobacterial DNA in 10 of 11 (91%) Mycobacterial containing samples (by gel analysis and hybridization). In addition, we have shown that the primers are able to amplify DNA from several species of Mycobacteria, but not from *E. coli*. Finally, we have shown that the new genus specific primer set is able to detect Mycobacteria to a level of less than 1 fg (0.2 Mycobacterial genome equivalents). These data make us believe that we have a promising set of primers for the detection of Mycobacteria in clinical samples.

## CONCLUSIONS

The data described above demonstrate that we have been able to design primer sets and probes for the sensitive detection of Mycobacteria and *M. tuberculosis* in clinical (sputum, etc.) samples. The results obtained for the *M. tuberculosis* primer set are generally similar to specificities obtained by other workers using PCR to detect Mycobacteria. Shawar et al (1993), using primers from the same insertion sequence as us, was able to correctly identify 55% of the *M. tuberculosis* samples by EtBr stained gels, and 74% by hybridization. Soini et al (1992) were able to detect 56% of *M. tuberculosis* containing samples on gels. Variables that may affect the sensitivity of the PCR assay include the method of lysis of the bacteria in the clinical sample, and the age of the sample. We are currently investigating methods of lysis of Mycobacteria in clinical samples, since there does not seem to be a standard protocol. In addition, Shawar et al (1993) have shown that long term storage of samples at 4°C greatly decreases the sensitivity of the PCR assay. One would expect that the stability of frozen samples would be significantly greater, though long term frozen storage of clinical samples can result in a notable decrease in the sensitivity of PCR.

While the genus specific primer set that we obtained from Dr. Patel does not appear to be sensitive enough for the routine detection of Mycobacteria from clinical samples, we have designed a new set of primers, based on the 16S rRNA sequences of Mycobacteria, that are much more sensitive. In a brief test of the primers in clinical samples, we were able to detect Mycobacteria in 91% of the samples tested, which we believe to be a promising beginning. We are testing more samples with this primer set to obtain a more accurate measure of its sensitivity and specificity.

We have yet to find a good set of primers for *M. avium*. We have tried two different primer sets derived from the IS901 sequence, with unsatisfactory results. Both sets examined appear to be specific, amplifying only *M. avium* DNA, and not amplifying DNA from *E. coli* or *M. tuberculosis*. Unfortunately, neither set tested is sensitive enough for the routine detection of *M. avium* in clinical samples, being able to detect only about 100 fg of DNA (about 20 Mycobacterial genome equivalents). We are in the process of designing other primer sets based on the IS901 sequence, as well as investigating methods for finding other DNA sequences unique to *M. avium*.

The results obtained for both genus specific primer sets, as well as for the *M. tuberculosis* primers are summarized in Table 3. As described above, these data imply that both the second, 16S rRNA derived primer set and the primers designed for *M. tuberculosis* show a great deal of promise for predicting the presence of Mycobacteria in clinical (mostly sputum) samples. We are continuing in the process of evaluating these primers by testing more clinical samples with known culturing results. In addition, we have 100 blinded samples from the WRAMC to be assayed . In the course of these assays, we will evaluate different means of isolating Mycobacterial DNA from clinical samples. After performing these experiments, we will be able to evaluate the sensitivity and specificity of the three primer sets on clinical samples. The estimated date of completion for these experiments will be 1 September, 1993. At that point, we should be ready to use the three primer sets in a prospective study assaying clinical samples for the presence of Mycobacteria.

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## Table 1: Summary Of PCR Testing Of Clinical Samples

| Sample # | Species (culture) | Mav17 (genus specific #1) | | Mav22 ( M. avium) | | M. tb primer set | |
|---|---|---|---|---|---|---|---|
| | | EtBr gel | Hybrid. | EtBr gel | Hybrid | EtBr gel | Hybrid. |
| 442 | M. gard. | - | + | - | - | - | - |
| 457 | M. xen. | - | - | - | - | - | - |
| 629 | M. tb. | + | +++ | - | - | + | + |
| 1020 | M. tb. | - | - | - | - | - | - |
| 1021 | M. tb. | ++ | ++ | - | +* | +++ | +++ |
| 1291 | M. gard. | - | - | - | +* | - | - |
| 1321 | M. tb. | - | - | - | - | ++ | - |
| 1330 | NG | - | - | - | - | - | - |
| 1455 | M. tb | ? | + | - | - | + | +++ |
| 1731 | NG | - | - | - | - | ++,- | -,++ |
| 1780 | M. tb. | - | - | - | - | - | - |
| 1832 | M. int. | - | + | - | + | - | - |
| 1833 | M. tb. | + | ++ | - | - | + | +++ |
| 1893 | M. int. | - | - | - | + | - | - |
| 1945 | M. tb. | - | - | - | | +++ | ++ |
| M1 | M. tb | - | + | nd | nd | ++ | ++ |
| M2 | M. tb. | - | - | nd | nd | + | ++ |
| M3 | M. tb. | - | + | nd | nd | +++ | +++ |
| M4 | M. tb. | - | - | nd | nd | +++ | +++ |
| M5 | M. tb. | - | - | nd | nd | + | +* |
| M6 | M. tb. | - | - | nd | nd | ? | + |
| 23 | M. tb. | + | + | - | - | +? | + |
| 2430 | M. tb | + | ++ | - | - | - | - |
| 2896 | M. tb. | - | +? | - | - | - | + |
| 2917 | M. tb. | - | - | - | - | - | - |
| 919 | M. tb. | + | ++ | | | ++ | +++ |
| 949 | M. tb. | + | ++ | | | - | +* |
| 1022 | M. tb. | - | + | | | - | - |
| 1034 | M. tb. | - | ?* | | | - | - |
| 1096 | M . tb. | - | - | | | - | + |
| 1385 | M. fort. | - | - | | | - | - |
| 1957 | Not proc. | - | + | | | - | + |
| 1038 | M. tb. | - | ++ | | | - | ? |
| 1046 | M. tb. | - | + | | | - | ? |
| 1047 | NG | - | - | | | ? | ? |
| 1068 | NG | - | - | | | - | ? |
| 1132 | M tb. | ++ | + | | | +++ | ++ |
| 1954 | M. tb. | - | -* | | | - | ++ |
| 1956 | M. tb. | - | -* | | | - | + |
| 1959 | MAI | - | ?* | | | ? | - |
| 2026 | M. tb(?) | - | -* | | | + | + |
| 2047 | M gard. | - | -* | | | - | - |

+,++, +++, ++++ Degrees of positive reaction
- Negative, * Some negative controls also lit up.
All samples were from WRAMC except the 6 samples labeled M1-M6, which are samples from NIH monkeys known to be infected with *M. tuberculosis*.

**Table 2: Summary of PCR Testing, Using new (16S rRNA) Genus Specific Primer Set (Genus #2)**

| Sample # | Species | Detection by EtBr | Detection by Hybridization |
|---|---|---|---|
| 7-21A | M. tb | + | + |
| 1134 | M. tb | + | + |
| 1742 | M. avium | + | ++ |
| 1782 | M. int. | - | - |
| 1879 | No growth | + | - |
| 1907 | M. tb | ++ | + |
| 7-21B | M. tb? | + | + |
| 810 | M. tb. | + | + |
| 812 | M. tb. | ++ | +++ |
| 848 | M. tb. | +++ | +++ |
| 849 | M. tb | +++ | +++ |
| 3550 | M. fort. | + | - |

**Table 3: Data Table for Sensitivity and Specificity**

| | PCR + | PCR - | |
|---|---|---|---|
| Culture + | TP | FN | TP+FN |
| Culture - | FP | TN | TN+FP |
| | TP+FP | TN+FN | TP+TN+FP+FN |

PCR detection of M. tuberculosis

| | PCR + | PCR - | |
|---|---|---|---|
| Culture + | 21 | 7 | 28 |
| Culture - | 1 | 9 | 10 |
| | 22 | 16 | 38 |

PCR detection of M. avium

| | PCR + | PCR - | |
|---|---|---|---|
| Culture + | 0 | 0 | 0 |
| Culture - | 4 | 14 | 18 |
| | 4 | 14 | 18 |

PCR detection of Mycobacteria (1st primer set)

| | PCR + | PCR - | |
|---|---|---|---|
| Culture + | 16 | 0 | 16 |
| Culture - | 13 | 4 | 17 |
| | 29 | 4 | 33 |

PCR detection of Mycobacteria (2nd primer set)

| | PCR + | PCR - | |
|---|---|---|---|
| Culture + | 9 | 0 | 9 |
| Culture - | 2 | 1 | 3 |
| | 11 | 1 | 12 |

Summary of PCR Results

| | M. tb. | M. avium | Genus1 | Genus2 |
|---|---|---|---|---|
| Sensitivity =TP/(TP+FN) | 75% | NA | 55% | 82% |
| Specificity=TN/(TN+FP) | 90% | 78% | 100% | 100% |
| Positive Predictive Value=TP/(TP+FP) | 95% | NA | 100% | 100% |
| Negative Predictive Value=TN/(TN+FN) | 56% | 100% | 24% | 33% |
| Efficiency=(TP+TN)/(TP+FP+TN+FN) | 79% | 78% | 60% | 83% |